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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Claude	Maina	West Newbury, MA
Jianping	Xiao	Wenham, MA
George	Tzertzinis	Cambridge, MA

Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Compositions and Methods for Generating Short Double-Stranded RNA Using Mutuated RNase III

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

[X] Customer Number: 28986

OR

[X] Firm or Individual Name	New England Biolabs, Inc.				
Address	32 Tozer Road				
Address					
City	Beverly	State	MA	Zip	01915
Country		Telephone	978-927-5054	Fax	978-927-1705

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Respectfully submitted,

SIGNATURE Harriet Strimpel

TYPED or PRINTED NAME Harriet M. Strimpel

TELEPHONE 978-927-5054

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REGISTRATION NO. 37,008 *(If appropriate)*

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PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number	NEB-238-B

INVENTOR(S)/APPLICANT(S)

Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Larry	McReynolds	Beverly, MA

Number 1 of 1

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Practitioner's Docket No. NEB-238-B *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Maina, et al.

Application No.: not yet assigned Group No.: N/A
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For: Compositions and Methods for Generating Short Double-Stranded RNA Using Mutated RNase III

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Docket No. NEB-238-B

IN THE UNITED STATES PATENT OFFICE AND TRADEMARK OFFICE
APPLICATION FOR UNITED STATES LETTERS PATENT

INVENTOR (S): Claude Maina
Jianping Xiao
George Tzertzinis
Larry McReynolds

TITLE: Compositions and Methods for Generating Short Double-Stranded RNA Using Mutated RNase III

ATTORNEY: Harriet M. Strimpel, Chief Patent Counsel
NEW ENGLAND BIOLABS, INC.
32 Tozer Road
Beverly, Massachusetts 01915

Customer No. 28986

(978) 927-5054; ext. 373

COMPOSITIONS AND METHODS FOR GENERATING SHORT DOUBLE-STRANDED RNA USING MUTATED RNASE III

Inventors: Claude Maina, Jianping Xiao, George Tzertzinis and Larry McReynolds

THIS IS A PROVISIONAL APPLICATION

BACKGROUND OF THE INVENTION

RNA interference (RNAi) employing short double-stranded RNA (siRNA) is a powerful tool for silencing gene expression (WO 01/29058, WO 01/68836, WO 01/75164). Large fragments of double-stranded RNA (dsRNA) elicit a non-specific response in mammalian cells through activation of the interferon (IFN) response pathway that leads to suppression of translation and cell death (Yang, et al., *Mol. Cell. Biol.* 21:7807-7816 (2001) and Wianny, et al., *Nat. Cell Biol.* 2:70-25, 25-33 (2000)). The standard method for generating siRNA is based on chemical synthesis of a pre-determined short sequence. In addition to the high cost of this method, there is no known method for predicting the short sequences effective for RNAi experiments and the users of the method resort to a "trial and error" approach.

A mixture of short lengths of double-stranded RNA obtained through partial digestion of large dsRNA with RNase III in the presence of magnesium ion buffer has been shown to "knock-down" the expression of cognate genes in cultured

mammalian cell lines via RNAi (Yang, et al., *Proc. Nat'l. Acad. Sci. USA* 99:9942-9947 (2002)). However, achieving partial digestions yielding the right size range of product is often a difficult and time-consuming process and requires gel separation to obtain fragments of the desired size. Furthermore the inclusion of all possible sequences contained in the starting material is not ensured. US Patent Application No. 10/622,240 herein incorporated by reference, describes how RNase III in the presence of transition metal ions can produce a heterogeneous mixture of fragments of a size suitable for gene silencing. This is a significant improvement on existing methods of making siRNA fragments. However, it would be desirable to circumvent the reliance on transition metal ions for forming RNA fragment mixtures enzymatically.

Summary

Present embodiments of the invention include an RNase III mutant characterized by a mutation in the position corresponding to E38 in *E.coli* RNase III. In one embodiment, the mutation is a point mutation. In a preferred embodiment, the mutation is a change in E to an alanine. In an alternative embodiment, a mutation is provided at a position corresponding to E65 in *E.coli* RNase III. The mutant RNase III is capable of cleaving more than 80% of a large dsRNA, of which at least 30% and more particularly at least 50% of the dsRNA is cleaved into fragments of 18-30 nucleotides (nt), more particularly fragments of a size 21-23 nt. The cleavage reaction is completed within 2 hours, more particularly less than 1 hour,

more particularly less than 15 minutes. However, the 18-30 nt fragments that result from cleavage are stable for greater than 2-5 hours and as much as 16 hours in the mutant RNase III reaction mixture.

Additional embodiments include a method of forming a preparation of dsRNA cleavage fragments having a size of 18-30 nt from a large dsRNA. The method includes the steps of (a) combining the preparation of large dsRNA molecules with a mutant RNaseIII in a reaction mixture; and (b) incubating the reaction mixture for less than 2 hours to form the dsRNA preparation which includes the dsRNA fragments having a size of 18-30 nt. The dsRNA preparation at completion of the reaction retains a substantially constant proportion of fragments of 18-30 nt for greater than 5 hours in the presence of the mutant RNase III. In particular embodiments of the invention, at least 30% of the dsRNA form fragments of a size of about 21-23 bp.

In a particular embodiment of the invention, the mutant RNase III of step (a) is an E38A mutant of an *E.coli* RNase III.

Additional embodiments provide a method for enzymatic cleavage of a large dsRNA to form a preparation of dsRNA fragments wherein at least 30% of the preparation comprises fragments of dsRNA having a size in the range of 18-30 nt. The method includes the steps of (a) adding a mutant RNase III to large dsRNA in the presence of magnesium ions to form a mixture; and (b) incubating the mixture of step (a) for less than

2 hours more particularly less than 1 hour, more particularly less than 30 minutes, more particularly less than 15 minutes to form a preparation of dsRNA fragments wherein at least 30% more particularly 50% of the large dsRNA comprises dsRNA fragments having a size of 18-30 nt.

In another embodiment of the invention, a method is provided for cleaving large dsRNA molecules of 50-100 nt enzymatically. The method includes the steps of: (a) adding a mutant RNase III to the target dsRNA to form a mixture; and (b) incubating the mixture for less than 2 hours to form a preparation of dsRNA fragments, wherein at least 30% of the fragments in the preparation have a size in the range of 18-30 nt.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an alignment of RNase III from *Aquifex aeolicus* and *E. coli*, highlighting regions and specific amino acids (boxed) of the protein thought to interact with dsRNA and be involved with cutting of the dsRNA (Blaszczyk, J., et al. *Structure* 9:1225 (2001)). Numbers indicate residue number of the amino acid to its right. Top row shows sequence of RNase III from *A. aeolicus*; second row shows sequence of wt RNase III from *E. coli*. All rows below show sequences of *E. coli* RNase III mutants constructed and assayed. Mutant residues are in italics. E. coli residues: 38, 45, 65, 117 correspond to *A. aeolicus* residues 37, 44, 64, 110, respectively.

Figure 2 shows the activity of E38A RNaseIII (E38A):

A His-tagged E38A RNqse III mutant was purified by standard Nickel resin affinity purification and assayed by standard methods. Digestion of 500 ng of substrate MalE dsRNA (900bp) with a serial dilution of E38A mutant RNase III in 50mM NaCl, 10mM TRIS HCL, 10mM $MgCl_2$, 1mM DTT pH 7.9 (NEB Buffer 2) (New England Biolabs, Inc., Beverly, MA) at 37°C, for 1 hr is shown.

Figure 2A. Cleavage of a dsRNA substrate was determined after adding mutant RNase III at concentrations of 0.03, 0.06, 0.125, 0.25, 0.5 , 1, 2, and 4 ug (lanes a-h) for one hour at 37C. The reaction mixtures were then electrophoresed through 20 % polyacrylamide gel and visualized via staining with Ethidium Bromide. In lane (f) the amount of 23 bp product of mutant RNase III digestion is ~60% of the dsRNA substrate

Figure 2B. Reactions c, f, g, h of 'A' were allowed to continue for 16 hrs followed by electrophoresis. The stability of the approximately 23 bp product was demonstrated to be similar at 1 hour and 16 hours.

Figure 2C. The cartoon depicts a current published model (Blaszczyk, J., et al. *Structure* 9:1225-1236 (2001)) of RNAse III/dsRNA complex. The *E. coli* RNase III enzyme is depicted as a dimer with amino acids E38, E65, E117 and D45 shown in contact with the dsRNA.

Figure 3 shows a time course reaction using 4 micrograms of E38A per 500 ngs of 900 bp MalE dsRNA (MalE dsRNA) in NEB Buffer 2 at 37°C. The reactions were stopped by the addition of EDTA (final concentration of 25 mM) after 1, 5, 10, 20, 30, 40 and 50 minutes and the products analyzed by gel electrophoresis. The digestion was substantially complete by 10 minutes even though the product was stable in the reaction mixture over the extended time period. Lane 1 (adjacent to the 1 minute incubation on the gel) contains synthetic 22 bp dsRNA size standard; lane 9 (last lane) contains undigested dsRNA.

Figure 4 shows a time course reaction using 4 micrograms of MalE dsRNA in NEB Buffer 2 at 37°C. Samples were removed at times indicated - 1 hour, 1 day, 2 days, 3 days, 4 days and 5 days and reactions stopped by the addition of EDTA (final concentration of 25 mM). The 23 bp product is present at each time point tested up to 5 days. This stability is greater than that observed for wt RNase III. Lane 1 (adjacent to the 1 hr time point) contains a synthetic 22 bp dsRNA size standard; lane 9 (last lane) shows 5 day mock-digested dsRNA.

Figure 5 shows that a 50nt dsRNA can be digested into approximately 23 bp fragments with RNase III. Synthetic 50 bp dsRNA (500 ngs) was digested with 4 micrograms of E38A for 1hr at 37°C followed by electorphoresis. Lane 1 - undigested 50 bp ds RNA. Lane 2 - 50 bp dsRNA digested with E38A for 1 hr. Lane 3 - synthetic 22 bp dsRNA size standard.

Figure 6 shows the reaction products using a His-tag purified E65A RNaseIII mutant which has been purified by standard Nickel resin affinity purification and assayed by standard methods. The enzyme reaction was done in NEB Buffer 2, at 37°C, for 1 hr using ds MalE RNA as a substrate .

Figure 6A. Cleavage of dsRNA was determined after 1 hour using a serial dilution of E65A RNase III having a concentration of 4 micrograms (lane h) to 0.03 micrograms (lane a). Reaction products were then analyzed on a 20% polyacrylamide gel.

Figure 6B. Reactions d, and f of 'A' were allowed to continue for 16 hrs followed by electrophoresis.

Figure 6C. Cartoon depicting a model of how *E.coli* RNase III is associated with dsRNA (Blaszczyk, J., et al. *Structure* 9:1225 (2001)). Arrows point to where residues are proposed to contact dsRNA.

Figure 7 shows a comparison of yields of 23nt ds RNA using different enzymes and buffers. All reactions used 0.5 micrograms of the same prep of MalE dsRNA. Other conditions followed the manufacturer's instructions except for E38A. E38A reaction conditions were: NEB Buffer 2, 37°C, 30 minutes for Protein Titer. Times of incubation were 1', 5', 10', 20', 30', 40', 50', 60'. "22" - 22 bp ds RNA; "-" - no enzyme reaction. Percents in specific lane designate product yield for those reactions - one data point only. One half of each reaction was loaded onto the gel.

Figure 7A - *E.coli* RNase III in a magnesium buffer (Ambion, Austin, TX).

Figire 7B - Human Dicer molecule from Stratagene, La Jolla, CA.

Figure 7C - *E.coli* RNase III in a manganese buffer (New England Biolabs, Inc., Beverly, MA).

Figure 7D - Mutant RNase III (E38A) in a magnesium buffer.

Figure 8 provides a summary of mutant activity as determined by the conditions described in Example II. '*' indicates mutants with wild type activity. Activities in parenthesis are those published for a genetic assay (Blaszczyk, J., et al. *Structure* 9:1225 (2001)).

DESCRIPTION OF THE EMBODIMENTS

We report the selective generation of dsRNA fragments of a length suitable for effective silencing of gene expression using digestions with RNase III mutants in the presence of standard buffers containing magnesium. Different types of mutants are described including: single point mutations altering RNA binding or cleavage residues and double point mutants. Examples of mutants are provided in the figures and examples that have comparable or improved activity to that described for wild type RNase III in a manganese containing buffer (US Patent Application No. 10/622,240).

The following terms as used in the description and in any accompanying claims have been defined below. These

definitions should be applied unless the context in which the terms are used requires otherwise.

"hsiRNA mixture" refers to a heterogeneous (h) mixture of short double-stranded RNA fragments containing at least one fragment (siRNA) suitable for silencing gene expression. The RNA fragments in the hsiRNA mixture consistently contain a substantial fraction (greater than about 20% of the total number of fragments in the reaction mixture without any purification step) having a length of 18-30 base pairs as determined by ethidium-stained native polyacrylamide gel analysis. The presence of fragments larger than 30 nt or smaller than 18nt is not excluded. The hsiRNA mixture is preferably obtained by digesting "large" double-strand RNA with RNAse III in the presence of divalent transition metal cations, preferably manganese ions.

"Silencing" refers to partial or complete loss-of-function through targeted inhibition of gene expression in a cell and may also be referred to as "knock down". Depending on the circumstances and the biological problem to be addressed, it may be preferable to partially reduce gene expression. Alternatively, it might be desirable to reduce gene expression as much as possible. The extent of silencing may be determined by any method known in the art, some of which are summarized in International Publication No. WO 99/32619 incorporated herein by reference. Depending on the assay, quantitation of gene expression permits detection of various amounts of inhibition for example, greater than 10%, 33%, 50%, 90%, 95% or 99%.

"Large double-stranded RNA" refers to any double-stranded RNA or hairpin having a double-stranded region greater than about 40 base pairs (bp) for example, larger than 100 bp or more particularly larger than 300 bp. The sequence of a large dsRNA may represent one or more segments of one or more mRNAs or the entire mRNAs. The maximum size of the large dsRNA is not limited herein. The double-stranded RNA may include modified bases where the modification may be to the phosphate sugar backbone or to the nucleotide. Such modifications may include a nitrogen or sulfur heteroatom or any other modification known in the art. The double-stranded RNA may be made enzymatically, by recombinant techniques and/or by chemical synthesis or using commercial kits such as MEGASCRIPT® (Ambion, Austin, TX) and methods known in the art. An embodiment of the invention utilizes HiScribe™ (New England Biolabs, Inc., Beverly, MA) for making large double-stranded RNA. Other methods for making and storing large dsRNA are described in International Publication No. WO 99/32619.

The double-stranded structure may be formed by self-complementary RNA strand such as occurs for a hairpin or a micro RNA or by annealing of two distinct complementary RNA strands.

"Heterogeneous" in the context of an hsiRNA mixture refers to double-stranded RNA fragments having non-identical sequences produced from a single large double-stranded RNA or

a mixture of large double-stranded RNAs after cleavage with RNase III or mutants thereof. The fragments collectively contain sequences from the entire length of the large RNA and hence form a heterogeneous mixture.

"RNase III" refers to a naturally occurring enzyme or its recombinant form and may include mutants and derivatives or homologs thereof. The utility of bacterial RNase III described herein to achieve silencing in mammalian cells supports the use of RNases from eukaryotes, prokaryotes viruses or archea in the present embodiments based on the presence of common characteristic consensus sequences. Embodiments of the invention do not preclude the use of more than one RNase to prepare an RNA fragment mixture. Any RNase can be used herein where the RNase contains the amino acid consensus sequence [DEQ][kRQT][LM]E[FYW][LV]GD[SARH] (PROSITE: PDOC00448 documentation for the RNase III). While not wishing to be bound by theory, it is here suggested that there is a region in an RNAse III of this type that specifically contacts substrate RNA. This region includes 4 specific amino acids and it is here shown that a mutation in at least one particular amino acid of this region results in increased activity of the RNase III for purposes of producing dsRNA fragments (Figure 1-8). Figure 1 shows a variety of mutations tested by applicants in different regions of the RNase III while Figure 8 describes how different mutants have wild type cleavage activity, are inactive, show reduced cleavage activity or display increased cleavage activity.

(The designation for the mutants are assigned by an amino acid position in a particular RNase III isolate. These amino acid positions may vary between RNase III enzymes from different sources. For example, E38 in *E.coli* corresponds to E37 in *Aquifex aeolicus*. The position E38 in *E.coli* and E37 in *A.aeolicus* correspond to the first amino acid position of the consensus sequence described above and determined by aligning RNase III amino acid sequences from the public databases by their consensus sequences. Embodiments of the invention are not intended to be limited to the actual number designation. Preferred embodiments refer to relative position of the amino acid in the RNase III consensus sequence).

Mutations in the RNAse III refer to any of point mutations, additions, deletions (though preferably not in the cleavage domain), rearrangements (preferably in the domain linking regions). Mutations may be at a single site or at multiple sites in the RNase III protein. Mutations can be generated by standard techniques including random mutagenesis and targeted genetics. Example 1 gives one approach to making mutants but this approach is not intended to be limiting.

Examples of mutants include E65A which produced a 23 bp product that differed from E38A with respect to stability of the enzyme and of the reaction product but was at least as effective as wild type RNase III in generating dsRNA fragments. Both mutants produced an increased yield of the 23 bp product as compared to wild type RNase III in Mg^{2+} buffer.

Where units are used to describe concentrations of RNase III in present experiments, the formula for conversion to weight/volume is 32 units = 1 µg/µl RNase III. Soluble mutant RNaseIII enzyme can be readily purified from recombinant sources as previously described in Example 2.

"Complete digestion" refers to an RNaseIII reaction in which fragments of double-stranded RNA of a size greater than about 30 base pairs (excluding digested material retained in the loading well or bound to enzyme) are no longer readily detectable on an ethidium bromide stained 20% polyacrylamide gel.

"Host cell" refers to cultured eukaryotic cells or cells in animals, including vertebrates such as mammals including humans, and invertebrates such as insects. Host cell also refers to cells from plants and microorganisms.

"Overlapping" refers to when two RNA fragments have sequences which overlap by a plurality of nucleotides on one strand, for example, where the plurality of nucleotides (nt) numbers as few as 2-5 nucleotides or by 5-10 nucleotides or more.

"Complementary sequence" refers to a sequence which is not necessarily 100% identical to a sequence to which it hybridizes but nevertheless is capable of hybridizing to a specified nucleic acid under stringent conditions where stringent conditions may include: 400mM NaCl, 40mM PIPES pH 6.4, 1mM

EDTA, 50°C or 70°C for 12-16 hours followed by washing. Sequence variations can be tolerated such as those that arise due to genetic mutation, strain polymorphism, evolutionary divergence or chemical modifications.

"Part or all" of a messenger RNA refers to that part of the messenger RNA (mRNA) which is complementary to a large dsRNA.

"Substantial portion" refers to the amount of sequence of a large dsRNA represented in sequences contained in an hsiRNA mixture. In one embodiment, the representative sequence is greater than 20%. In other embodiments, the representative sequence may be greater than 30%, 40%, 50%, 60%, 70%, 80% or 90%.

"One or more dsRNAs" refers to dsRNAs that differ from each other on the basis of sequence.

"Target gene or mRNA" refers to any gene or mRNA of interest. Indeed any of the genes previously identified by genetics or by sequencing may represent a target. Target genes or mRNA may include developmental genes and regulatory genes as well as metabolic or structural genes or genes encoding enzymes. The target gene may be expressed in those cells in which a phenotype is being investigated or in an organism in a manner that directly or indirectly impacts a phenotypic characteristic. The target gene may be endogenous or exogenous. Such cells include any cell in the body of an adult or

embryonic animal or plant including gamete or any isolated cell such as occurs in an immortal cell line or primary cell culture.

The term "stable preparation" is used here and in the claims to describe a preparation of target dsRNA which is cleaved in the presence of a mutant RNaseIII where at least 30% of the starting material is cleaved into fragments having a size 18-30nt more particularly 21-23 nt and the cleavage product is stable for more than 2 hours, more particularly stable for 5 hours more particularly stable for 16 hours where stability is determined by little or no detectable change in the size profile on a gel of the reaction mixture at different times after the RNaseIII has been added to the dsRNA substrate.

The introduction of an hsiRNA mixture into vertebrate, invertebrate, plant or protoplast cells, or micro-organisms may be achieved directly into the cell or introduced extracellularly into a cavity or interstitial space, into the circulation of an organism, orally, by bathing, transdermally, by a transmucosal route, topically or by use of viral vectors to infect the host with the DNA.

Standard protocols of transfection or transformation may be used for introducing dsRNA into cells in culture, for example, protocols using Lipofectamine 2000, oligofectamine (Invitrogen, Carlsbad, CA), TRANS-IT TKO® (Mirus Corp., Madison, WI), Targefect (Targeting Systems, Santee, CA), calcium phosphate or electroporation. Engineered vectors containing fragments from hsiRNA or siRNA can include bacterial vectors, plasmids or

viral vectors for transforming or transfecting whole organisms. A gene gun may be utilized for plants for directing dsRNA into chloroplasts for example. The methodology for introducing foreign nucleic acids into organisms and cells is well known in the art. Introduction of the dsRNA mixture of DNA clones expressing individual fragments from a particular dsRNA mixture into whole animals can be achieved by means of standard techniques for introducing nucleic acids.

In this specification and the appended claims, the singular forms of "a", "an" and "the" include plural reference unless the context clearly dictates otherwise.

Advantages of the methods described herein include:

(a) obtaining rapidly (within minutes) an enhanced concentration of dsRNA fragments of a size suitable for silencing of gene expression by a rapid, cost effective process that is not dependent on a gel based size separation step. The methodology provides dsRNA fragment preparations from large dsRNA that contain a plurality of overlapping dsRNA fragments in which less than about 20% are uncut large dsRNA and more about 30% have a fragment size of 18-30 base pairs more particularly 21-23 bp. In preferred embodiments of the method, as much as 60% of the large dsRNA is cleaved into fragments of 21-23 nt. Because of its simplicity, this approach is amenable to automation and high throughput;

(b) forming a preparation of dsRNA fragments with gene silencing activity without requiring identification of the particular fragment giving rise to the gene silencing effect;

(c) providing a means to utilize the products of the method by cloning individual fragments or forming libraries or arrays of clones to enable mapping these fragments with respect to the RNA from which they are derived as well as testing individual fragments for gene silencing activity;

(d) providing dsRNA reagents for applications which include: silencing single genes or families of genes in a eukaryotic cell or organism to study function using standard transfection or transformation techniques for nucleic acids;

(e) reduction of target effects compared with synthetic dsRNA preparations; and

(f) using these dsRNA fragments as therapeutic agents or in therapeutic agent screening or target validation assays.

The advantages of RNase III mutants such as E38A described herein further include: (i) the ability to obtain the desirable size range of dsRNA products generated by substantially complete digestion of larger dsRNA molecules corresponding to a large portion or the total sequence of the target mRNA so as to circumvent the need for selecting an effective target short sequence (ii) incubation in standard buffers to facilitate making dsRNA and cleaving it to the desired size all

in a single reaction vessel; (iii) enhanced yield of fragments in the desired size range; (iv) enhanced stability of the fragments obtained using mutant enzymes compared with wild type RNase III in a standard Magnesium buffer; (v) flexibility in time of incubation where a complete reaction can be achieved in as little as 10 minutes although longer incubation times are not detrimental to obtaining the desired reaction products; (vi) reduced cost of gene silencing reactions; (vii) opportunities for multiplexing reactions and for generating libraries of fragments; (vii) the ability to cleave into 23bp fragments, double stranded RNA having a size as small as 50 bps; and (viii) the ability to generate 23 bp fragments from larger dsRNA in vivo (cell culture or whole organism).

The fragmentation of large dsRNA molecules (greater than about 40 bp to at least 10kb) including linear dsRNA or hairpins, provides a population of short RNAs which include multiple effective short sequences (18-30 bp) corresponding to the mRNA for silencing.

Additionally, the advantages of the method described herein obviate the need for calibration of the time of digestion or the amount of enzyme used (Figures 2,3,4 and 6), and further eliminate the need to remove undesired digestion products by gel electrophoresis or other tedious separation methods making the method amenable to automation and suitable for high throughput formats. The RNA starting material can be readily obtained by *in vitro* enzymatic transcription (NEB catalog) or

chemical synthesis and can be a double-stranded molecule or an ssRNA that forms a double stranded hairpin.

A substantial portion of the dsRNA fragments after digestion with mutant RNase III have a size in the range of 18-30 bp, more particularly 21-23 bp suitable for gene silencing in cultured mammalian and insect cells. It is expected that these fragments will also be active in gene silencing in whole organisms such as, plants, microorganisms and animals including humans as well as to cultured cells from the same.

The digestion of large dsRNA preferably results in at least 30% of the preparation having the desired fragment size. More particularly, the desired percentage achieved with mutant RNase III is greater than 40% more particularly greater than 50%. In a preferred embodiment, the E38A mutant is capable of digesting as much as 60% of the substrate to completion within 10 minutes at 37°C in standard buffer (NEB buffer 2). In this preferred embodiment, the yield of RNA fragments from digestion of large dsRNA with RNase III E38A mutant results in about 2X the yield obtained with RNase III (wt) in the presence of manganese ions and about 10X the yield obtained with RNase III (wt) in the presence of magnesium ions.

One of the problems in the field of gene silencing is that of identifying a short dsRNA (15-30 bp) that can achieve the desired goal of effectively targeting a particular messenger RNA for cleavage. In embodiments of the invention, this problem is solved by utilizing a large dsRNA having a sequence that is

identical to all or part of the target mRNA including untranslated mRNA and cleaving this large RNA into multiple overlapping fragments of the appropriate size for gene silencing. It is here asserted that the cleavage products are representative of the entire length of the large dsRNA and that the dsRNA fragment preparation is capable of gene silencing by transfecting a variety of cells including insect cells and mammalian cells. Importantly, off-target are minimized by the use of hsiRNA mixtures (Example IV).

Once a dsRNA fragment mixture is obtained, it is possible to make a library of clones containing DNA sequences corresponding to individual dsRNA fragments in the mixture. When provided with appropriate promoters, individual clones or mixtures thereof can be used to transfect cells so as to provide a continuous supply of the short dsRNA for use in long-term gene silencing. Silencing of gene expression as a result of transfection of an individual clone or selected mixtures of clones into a target cell or organism may have particular advantages in for example, therapeutic applications, over transient gene silencing effects achieved by transfecting cells with the dsNA itself. This provides new reagents for therapeutic applications providing an unlimited supply of an agent that specifically modulates gene expression of a particular gene.

Other advantages of obtaining clones of individual fragments, as described herein, include (a) a resource for understanding which single fragment or subset of fragments in a mixture of fragments formed by cleavage of dsRNA is capable of

gene silencing while other fragments in the mixture are not; (b) a means of studying why some RNA fragments are efficacious in gene silencing and others are not; (c) establishing the specificity of a particular hsiRNA for a particular mRNA; (d) establishing the unique characteristics of an hsiRNA mixture from a particular RNase III versus a different RNase III, and (e) characterizing the site at which hsiRNA induces cleavage on a target mRNA; and (f) the generation of computer algorithms for the design of synthetic siRNA based on statistical analysis of the cloned fragments.

Specificity of gene silencing

Specificity of gene silencing for a particular targeted mRNA can be confirmed using a BLAST analysis of sequences in the targeted mRNA to determine that no extended regions in the RNA (over 20 bases long) are identical to other gene transcripts to avoid non-specific gene silencing.

Using the methods described herein, RNA preparations that are specific for a single member of a gene family and do not silence mRNA from other members of that gene family can be prepared from long dsRNA that is complementary in sequence to a segment of the target mRNA (also referred to as long dsRNA segments). Alternatively, RNA preparations can be prepared that have specificity for any gene in a gene family but do not have specificity for other genes outside the gene family.

The appropriate gene silencing effect may be achieved by targeting mRNA sequences that are unique or that form part or all of a consensus region for a family of mRNAs.

A "super potent" mixture of RNA fragments may be prepared according to the present methods in which individual RNA fragments that have been optionally cloned and have been identified as triggering cleavage at a site on the target mRNA are combined to obtain a mixture with the desired gene silencing effect.

One of the advantages of present embodiments is the ability to rapidly prepare a mixture of RNA fragments that can be tested *in vivo* for activity and from which subsets of fragments having particular sequence specificities can be selected as desired without the need for expensive chemical synthesis of oligonucleotide fragments or the more haphazard approach offered by partial enzymatic digestion or by crude extracts of cells containing naturally occurring DICER. A benefit of the RNase III digest described herein is that the entire large dsRNA is substantially represented by overlapping fragments.

Insights into gene silencing can be achieved by varying the size and sequence characteristics of the large RNA with respect to the target mRNA that is expressed from the template DNA. For example, serially deleted or randomly cut DNA templates can be used for the generation of variable size dsRNAs, which upon digestion with RNase III as described herein, can be tested for effectiveness in silencing.

An assay which may be readily used to determine whether a mutant RNase III has improved cleavage activity includes an *in vivo* and an *in vitro* test as follows:

(1) *In vivo assay*: dsRNA having a sequence complementary to a transcript of a marker protein is subjected to RNase III digestion to produce a dsRNA fragment preparation. Host cells normally expressing the marker protein are transfected with the RNA fragment preparation and changes in the expression of the marker phenotype determined to ascertain whether a knock down effect in gene expression has occurred as a result of the transfection with the RNA fragment mixture. The in vivo assay has been described in more detail in US patent application 10/622,240 incorporated herein by reference.

(2) *In vitro* assay: dsRNA is subjected to RNase III digestion and the digest run on acrylamide gels to determine the size of fragments produced at various times of incubation and in selected buffers.

The simplicity of testing hsiRNA from different segments provides a rapid primary screening of a target sequence for determining the activity of siRNA molecules in a heterogeneous siRNA mixture.

The methods described herein can also be applied to producing multiple dsRNA fragment mixtures which can then be used to simultaneously silence multiple genes. Additional uses

include targeting upstream or downstream regulatory regions with dsRNA to modulate expression. Accordingly, a mixture of large dsRNA obtained by transcription of a collection of DNA templates (see NEB catalog) can be digested by mutant RNase III in the presence of magnesium ions.

The above described generation of dsRNA fragment mixtures or clones thereof for making selected siRNA fragments can be achieved in part or as a whole by utilizing a kit of the type described in Example IV. Instructions are provided for making a desired large dsRNA, for generating dsRNA mixtures and for transfecting cells with such mixtures. Example IV describes how individual fragments in these mixtures may then be cloned and their sequences analyzed and mapped.

Site-specific cleavage of Target mRNA

As described herein, the set of dsRNA fragments produced by cleavage of large dsRNA with mutant RNase III in the presence of Magnesium is a heterogenous mixture of overlapping fragments. This mixture is capable of silencing a gene presumably by cleaving a mRNA transcript of a target gene where the large dsRNA is complementary to sequences in the mRNA. Analysis of the dsRNA mixtures produced, permits the characteristics of the most effective target sequences to be defined with single nucleotide resolution.

Another advantage of present embodiments is that once a single siRNA fragment or specific mixture or subset of dsRNA

fragments are obtained, they can be cloned as described in to provide a continuous or *in vivo* regulated supply of these nucleic acids without the need for *de novo* synthesis for each experiment. Alternatively, the siRNA can be incorporated into the genome of an RNA virus for example, the lentivirus, for delivery into the host cell.

Illustrative Uses

The availability of cloned fragments provides not only a continuous supply of a reagent or therapeutic agent but also a novel therapeutic approach in which a desired knockdown effect can be maintained by gene therapy techniques in a whole organism without repeated administration of the siRNA fragment. Clones expressing siRNA fragments or dsRNA mixtures can be used for complete, modulated or transient *in vivo* silencing of a target gene.

A gene derived from any pathogen can be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen or maintenance of the infection. The inhibitory RNA could be introduced in cells *in vitro* or *ex vivo* and then subsequently placed into an organism to effect therapy, or the organism could be directly treated by *in vivo* administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for

treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

Embodiments of the present invention may be applied to treatment or development of treatments for cancers of any type, including solid tumors and leukemias, examples of which are listed in International Publication No. WO 99/32619.

Embodiments of the present invention are exemplified as follows. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

All references cited herein are incorporated by reference.

EXAMPLES

Example I: Preparation of E.coli RNase III mutants

All *E. coli* RNAse III mutants (except for E117D which was generated by random mutagenesis using PCR) were constructed by a standard 2 step PCR sewing technique (Methods Enzymol. 185, 60-89, 1990). Other cloning techniques used herein are

standard in the art and can be found in Sambrook, J., Fritsch, E. F., Maniatis, T. "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. The starting plasmid was E. coli RNAse III cloned into pET16B (EMD Bioscience, Inc., San Diego, CA) which produces a His-tagged RNAse III protein under control of a T7 promoter.

WT RNAse III

E. coli RNAse III was amplified from a pMalE/RNAse III clone with the following primers:

SEQ 1 - ACAGGATCCCATGAACCCCATCGTAATTAAT

SEQ 2 –ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT

The PCR product was cleaved with BamHI and cloned into the BamHI site of pET16b, resulting in a plasmid that synthesizes His-tagged WT RNAse III.

Formation of E38A mutant

The primers used to construct the carboxy terminal half of *E.coli* RNase III (Accession No. X02946) with an E38A mutation were: CAGTAAACATAACGCGCGTTTAGAAT and primer SEQ 2-ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT

The primers used to construct the amino terminal half of RNAse III with an E38A mutation were: SEQ 3 AATTCTAAACGCGCGTTATGTTTACT and NEB primer cat# 1248 (New England Biolabs, Inc., Beverly, MA).

The two PCR products were then 'sewn' together in one PCR reaction using both as substrates and NEB primers 1228 &

1248 as primers for the reaction. The resulting product was then cloned back into pET16b at a XbaI site, resulting in a His-tagged RNAse III with an E38A mutation.

Formation of E65A RNase III

E65A RNAse III was constructed in a two-step process. In the first step the above plasmid was amplified with the following primers in two PCR's:

SEQ 4 – TCCGGCTCATATCGCCTGCATCCACACGAGGGA

SEQ 2- ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT

SEQ 5 – CTCGTGTGGATGCAGGCGATATGAGCCGGAT

The two PCR products were then used as substrates in a subsequent PCR reaction with the following primers:

Primer NEB cat No. 1248 and SEQ 2-ACAGGATCCTCTAGAGTCATTCCAGCTCCAGTTTTT

The resulting PCR product was cleaved with XbaI and cloned into the XbaI site of the modified pET16b vector described in I.B.

Formation of additional RNAse III mutants

Any specific point mutation can be constructed from the cloned wt RNAse III gene by a similar method to the one described to create both the E38A and E65A mutants. In the first step two primers, one for the coding strand and one for the non-coding strands, are designed such that they introduce the desired mutation at the selected amino acid. The primers are of sufficient length and span such that they will still anneal to the substrate given the mismatch necessary to introduce the

mutation. The primer for the coding strand is matched with an appropriate downstream primer on the opposite strand in PCR that amplifies this region of the RNAse III gene. A similar PCR is performed using the non-coding primer and an appropriate primer upstream on the opposite strand that results in an overlapping upstream fragment of the RNAse III gene. The two fragments are 'sewn' together using the two fragments as substrates and the outside primers. The resulting RNAse III gene now contains the point mutation desired and can be cloned back into the appropriate vector.

The E38 residue of the *E. coli* RNAse III gene corresponds to the E37 residue of the *Aquifex aeolicus* RNAse III gene. Likewise D45 corresponds to D44, E65 corresponds to E64, E117 to E110. This was determined by several standard multiple sequence alignment software packages. The corresponding amino acid residues can be determined for any RNAse III gene by similar methods.

Example II: Production and purification of RNase III mutants

Expression & Purification

30 ml cultures of each mutant and WT clones were grown in *E. coli* ER2566 (New England Biolabs, Inc., Beverly, MA) to mid log phase, then induced by the addition of IPTG to a final concentration of 100 mM and shaken at 15°C overnight. Induced cultures were lysed by sonication.

The RNase III mutants were purified from the cleared lysates by Qiagen Nickel resin affinity purification (according to Manufacture's instructions) and assayed by standard methods.

The enzyme reaction was performed in NEB Buffer 2, at 37°C, for 1 hr using 500 ngs of a 900 bp ds RNA as a substrate. The product of the reaction was analyzed by polyacrylamide electrophoresis.

RNase Activity Assay

1 ug of MalE dsRNA was digested with RNAse III (wt and mutants in the range consistent with optimal conditions shown in Figure 2) in a 20 μl reaction mixture at 37°C in NEB Buffer 2 (0.1 M NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MnCl_2$, 1 mM dithiothreitol) for 1 hour.

As can be seen in the Figure 2A, the end product of digestion of dsRNA with E38A is predominantly a dsRNA of 23 bps in length (lanes f, g, and h). This product appears in as little as 1 minute and the reaction is 'complete' by 10 minutes (Figure 3). In addition the 23 bp product is stable for at least 16 hrs (Figure 2B, lane h) and is slowly lost over several days (Figure 4).

The activity of E65A (Figure 6) is in direct contrast to what was reported in (Blaszczyk, J., et al. *Structure* 9:1225-1236 (2001)). In this paper the authors describe an E65A mutation of RNase III as disabling RNAse III function. By our assay, E65A produces the same product as E38A (Figure 6A, lanes f-h). However, the product appears to be less stable and has disappeared by 16 hrs (Figure 6B, lanes d & f).

Example III: DsRNA cleavage and Gene Silencing Activity in cultured cells.

To test the ability of the dsRNA product of RNase III mutant digestion to induce RNA interference-an in vivo assay according to US patent application 10/622240 was carried as follows: dsRNA made from firefly luciferase is cleaved with the E38A or E65A mutant RNase IIIs (Figure 1); the dsRNA product is then purified by ethanol precipitation. Drosophila Schneider S2 cells are transfected with a reporter plasmid expressing the firefly luciferase gene, another reporter acting as a transfection control and dsRNA. Cells transfected with the reporter and no dsRNA show significant luciferase activity. Cells transfected with the reporter and full-length (approximately 1kB) luciferase dsRNA show a decrease in luciferase activity. There is no effect when GFP dsRNA is used and there is a significant decrease in luciferase activity when the luciferase dsRNA cleaved by the RNase III mutant is used.

Example IV: The cleavage products of E38A RNase III on dsRNA is a set of heterogeneous fragments that are overlapping.

Short dsRNA cleavage products of mutant RNase III digestion contain sequences representing the entire parent sequence. This is determined by cloning and sequencing the dsRNA cleavage products. Generation of a library of cloned RNase III products is readily achieved. The techniques used here to demonstrate the presence of overlapping fragments are

the same as those described in US 10/622,240 herein incorporated by reference.

Example V: Kits for generating hsiRNA and for gene silencing in mammalian cells

A kit is provided for *in vitro* generation of dsRNA mixtures and optionally for transfection of RNA fragments into mammalian cells.

In an embodiment of the invention, each kit contains reagents for processing multiple large dsRNAs for transfections in a 24-well plate format (sufficient for 100 transfections) and includes instructions for use.

Kit Components

The kit may contain enzyme and at least one of vectors, primers and buffers. Examples of components in a kit, all of which are individually available from New England Biolabs, Inc. (Beverly, MA), are listed below.

T7 RNA Polymerase, 150 units/µl,	25 µl
10X Buffer/NTPs (see formulation below)	60 µl
30X High Molecular Weight Component Mix (HMW) (see formulation below)	20 µl
BT7-minimal Primer (19 MER), 5′-Biotin-dCTCGAGTAATACGACTCACTATAG-3′, (SEQ ID NO:11) (10 µM)	25 µL
10X Mutant Ribonuclease III (1.4 µg/µl)	100 µL
10X hsiRNA Buffer (see formulation below)	
10X $MgCl_2$ (200 mM)	1000 µL

10X EDTA (250 mM)	1000 µL
Litmus 38iluc control template,	1 µg
RNase-free glycogen 10 µg/µL	50 µL

Plasmid DNA 500 µg/ml in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)

In addition, the kit may include transfection reagents, RNA size markers and Streptavidin-coated beads.

Buffer compositions

(a) 10X Buffer/NTPs:
400 mM Tris-HCl, pH 8.1
190 mM $MgCl_2$
50 mM DTT
10 mM spermidine
40 mM each NTP

(b) 30X High Molecular Weight (HMW) Mix:
20 mM Tris-HCl, pH 8.1
1.5 mg/ml BSA
100 units/ml inorganic pyrophosphatase (yeast)
12,000 units/ml pancreatic ribonuclease inhibitor
50% glycerol

(c) 10x hsiRNA buffer
0.5M Tris-HCl, pH 7.5
10mM DTT

The kit utilizes mutant RNase III in an optimized buffer to produce fragments in the range of about 18-30 nt (more particularly 21-23 nt) from long dsRNA. The dsRNA product is cleaved with mutant RNase III to reproducibly yield dsRNA mixtures suitable for silencing gene expression. The sequences of different siRNA fragments in the mixture map to sequences along the entire target gene. The dsRNA mixtures can be purified by ethanol precipitation and used in transfection.

In addition to mutant RNase III, the kit may include reagents for high-yield *in vitro* transcription of large dsRNA from DNA templates flanked by T7 promoters along with instructions for use and optionally a reaction vessel for conducting the reactions.

An example of instructions accompanying the kit include the following:

(1) Cloning the DNA template prior to *in vitro* transcription to generate dsRNA

One approach to making a DNA template for transcription is to clone a DNA of interest in Litmus 28i/38i bi-directional transcription vectors (New England Biolabs, Inc., Beverly, MA). The DNA of interest can then be amplified by PCR using a single T7 promoter-specific primer such as a BT7 Minimal Primer which produces a linear product with the target sequence flanked by T7 promoters which define the ends.

Alternatively target gene-specific primers with appended T7 promoters can be used to amplify any specific cDNA sequences. For example, the amplification primer:

5'TAATACGACTCACTATAG**aaggacagatggttaagtac**-3'
T7 promoter (SEQ ID NO:12)

in which a T7 promoter (underlined) located at the 5′ end preceding the target-specific sequence (bold) can be used for amplifying any cDNA template.

Biotinylated BT7 primer can be used to amplify any sequence flanked by T7 promoters. Optionally, the amplified biotinylated DNA template can be isolated by binding to streptavidin magnetic beads (New England Biolabs, Inc., Beverly, MA) and used directly as a template for transcription. For forming an immobilized DNA template, 25-50 µL of streptavidin magnetic bead suspension is added to the amplification (PCR) reaction mix with an equal volume of 1 M NaCl and incubate at room temperature for 10-15 minutes. The supernatant is removed in the presence of a magnet and the beads washed with 0.5 mLs TE, 0.5 M NaCl. The resuspended beads can be used directly in the transcription reaction. *In vitro* transcription of the immobilized DNA template produces DNA-free double-stranded RNA.

Amplification can be achieved by any polymerase dependent method such as PCR. The amplification product is purified by ethanol precipitation, or by a chromatographic method (e.g., QiaQuick® column (Qiagen, Studio City, CA)) and resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, prepared with Milli-Q water or equivalent) to a final concentration of ~500 µg/ml.

A control consisting of GL3 luciferase can be prepared using a Litmus 38iLuc plasmid in which a 1.0-kbp fragment of

the GL3 luciferase gene is cloned in the SphI and NgoMIV sites of Litmus 38i. Linearization with MfeI and StuI (in separate reactions), followed by *in vitro* transcription of the combined linearized templates, produces double-stranded RNA 1.0 kbp in length.

Pilot studies can be undertaken for providing an hsiRNA mixture for specific gene silencing using one or more fragments obtained by cleaving double-stranded RNA having a length of 100-600 bp including RNA derived from restriction fragments of a cDNA which has been subcloned into Litmus28i/38i vectors (New England Biolabs, Inc., Beverly, MA).

In vitro Transcription

In vitro transcription is performed using the DNA template prepared as described above. The volume of template used in the transcription reaction depends on the method of purification. For unpurified PCR product, no more than 5 µl is used per 30 µl reaction. The amount of added template DNA should not exceed 1 µg per 30 µl reaction.

RNase-Free Water	50 - x µl
10X Buffer/NTPs	6 µl
DNA template (~0.5-1 µg)	x µl
30X HMW Mix	2 µl
T7 RNA Polymerase (150 U/µl)	2 µl
	60 µl

Incubation at 42°C can improve yields of RNA transcripts containing substantial secondary structure. As it is difficult to

gauge the secondary structure content in a particular transcript, we recommend that all transcription be carried out at 42°C if possible. Transcription yields increase linearly for the first 90 minutes (approximately) and reach maximum after 2-3 hours. Reactions can be carried out overnight if desired, but yields will not be higher. Double-stranded RNA is stable upon prolonged incubation at 37°C.

The transcription reaction can be analyzed on a 1% agarose gel taking care to avoid RNase contamination. Double-stranded RNA migrates approximately as the DNA template used in the reaction. The expected length of the transcript from the Litmus 38iluc control template is 1000 bp.

The double-stranded RNA transcription product is purified by ethanol precipitation. One-tenth volume of 3 M NaOAc is added at a pH 5.5 with 2 volumes of cold 95% ethanol. Incubate on ice for 15 minutes, or store at −20°C overnight. Spin for 15 minutes in a microcentrifuge at 14,000 rpm. Remove supernatant, add two volumes 80% ethanol, incubate at room temperature for 10 minutes, centrifuge for 5 minutes, and decant and drain the tube. Allow the pellet to air-dry. Dissolve the dried RNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, or dH_20.

Forming an dsRNA mixture

Use 1X (10-fold diluted) RNase III at a concentration of (0.14 ug/ul) and 0.07 µg/µL of dsRNA in the digestion reaction as in the following example.

Combine the following:

dH_2O	105 - x µL
10X hsiRNA Buffer	15 µl
dsRNA	x µL (10 µg)
mutant RNaseIII	15 µl
10X $MgCl_2$	15 µl
	150 µl

Incubate for 10 min at 37°C.

Promptly add 15 µl 10X EDTA to stop the reaction.

For monitoring the products of digestion, a 10-20% native polyacrylamide gel is suitable. The product of digestion reveals that the long dsRNA has been cleaved to yield an dsRNA mixture of fragments having a size in the range of 18-25 nucleotides regardless of the length of the starting long dsRNA. The mixture can be purified by the single step of ethanol precipitation prior to use in transfection.

Ethanol precipitation of hsiRNA fragments

Add one-tenth volume of 3 M NaOAc, pH 5.5, 2 µL glycogen solution and 3 volumes of cold 95% ethanol. Place at −70°C for 30 minutes, or −20°C for 2 hrs-overnight. Spin for 15 minutes in a microcentrifuge at 14,000 rpm. Remove supernatant carefully avoiding the small pellet, add two volumes 80% ethanol, incubate at room temperature for 10 minutes, centrifuge for 5 minutes, and decant and drain the tube. Allow

the pellet to air-dry. Dissolve the dried RNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, or dH_20.

Determining dsRNA concentration

This can be measured using a UV spectrophotometer (OD at 260 nm of 1 corresponds to 40 µg/mL dsRNA) or a fluorometer (using RIBOGREEN®, Molecular Probes, Eugene, OR) or comparisons to siRNA standards used in the art.

Transfection guidelines

After ethanol precipitation, dsRNA mixtures can be directly transfected into mammalian cells using reagents and protocols suitable for oligonucleotide transfections such as lipofectin 2000, oligofectamine, TRANS-IT TKO® (Mirus Corp., Madison, WI) and Targefect (Targeting Systems, Santee, CA). Additionally Calcium Phosphate and Electroporation have been reported to be efficient in transfecting short RNAs.

Amounts of 25-100 ng of dsRNA can be used per transfection well (24-well format) as an initial amount to be adjusted according to experimental findings.

Large dsRNA may be synthesized by *in vitro* transcription as described above using a modified transcription buffer containing modified ribonucleotides such in place of NTPS in 10X bufffer described above such as 2-fluoro-ribo-CTP, 2-fluoro-ribo-UTP, 2-O-methyl-ribo-CTP, 2-O-methyl-ribo-UTP, 2-O-methyl-

ribo-ATP, 2-O-methyl-ribo-GTP or other 2' modifications that render the dsRNA more stable or resistant to degradation. A DURASCRIBE® kit (Epicentre Technologies, Madison, WI) may be used for these purposes.

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What is claimed is:

1. An RNase III mutant, characterized by a mutation in the position corresponding to E38 in *E.coli* RNase III in which the E has been mutated to an alanine.

2. An RNase III mutant according to claim 1, capable of cleaving more than 80% of a large double-stranded RNA (dsRNA), into fragments in which more than 30% have a size of 21-23 nucleotide (nt).

3. A method of forming a preparation of dsRNA cleavage fragments having a size of 18-30 nt from a large dsRNA molecule, comprising:

(a) combining the dsRNA with a mutant RNase III in a reaction mixture; and

(b) incubating the reaction mixture for less than 2 hours to form the preparation of dsRNA cleavage fragments wherein at least 30% of the dsRNA cleavage fragments have a size of 18-30 nt, and wherein the percentage of the dsRNA cleavage fragments of size 18-30 nt remains substantially constant for greater than 5 hours in the presence of the mutant RNase III.

4. A method for enzymatic cleavage of a large dsRNA to form a preparation of dsRNA fragments in which at least 30% have a size in the range of 18-30 nt, the method comprising:

(a) adding a mutant RNase III to target dsRNA in the presence of magnesium ions to form a mixture; and

(b) incubating the mixture of step (a) for less than **2** hours to form a preparation of dsRNA fragments wherein at least 30% have a size of 18-30 nt.

5. A method according to claims 1-4, wherein the large dsRNA has a length in the range of 50-100 nt.

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Figure 1

E. coli RNAse III Mutants

Aquifex aeolicus	37	E	T	L	E	F	L	G	D	A	63	R	E	G	F	L	S	107	D	V	F	E	A	L
E. coli	38	E	R	L	E	F	L	G	D	S	64	D	E	G	D	M	S	114	D	T	V	E	A	L
E38Q	38	*Q*	R	L	E	F	L	G	D	S	64	D	E	G	D	M	S	114	D	T	V	E	A	L
E38A	38	*A*	R	L	E	F	L	G	D	S	64	D	E	G	D	M	S	114	D	T	V	E	A	L
D45A	38	E	R	L	E	F	L	G	*A*	S	64	D	E	G	D	M	S	114	D	T	V	E	A	L
D45V	38	E	R	L	E	F	L	G	*V*	S	64	D	E	G	D	M	S	114	D	T	V	E	A	L
E65P	38	E	R	L	E	F	L	G	D	S	64	D	*P*	G	D	M	S	114	D	T	V	E	A	L
E65A	38	E	R	L	E	F	L	G	D	S	64	D	*A*	G	D	M	S	114	D	T	V	E	A	L
E117D	38	E	R	L	E	F	L	G	D	S	64	D	E	G	D	M	S	114	D	T	V	*D*	A	L
E38Q,E65P	38	*Q*	R	L	E	F	L	G	D	S	64	D	*P*	G	D	M	S	114	D	T	V	E	A	L
E38A,E65A	38	*A*	R	L	E	F	L	G	D	S	64	D	*A*	G	D	M	S	114	D	T	V	E	A	L

Figure 2

RNAse Activity of E38A

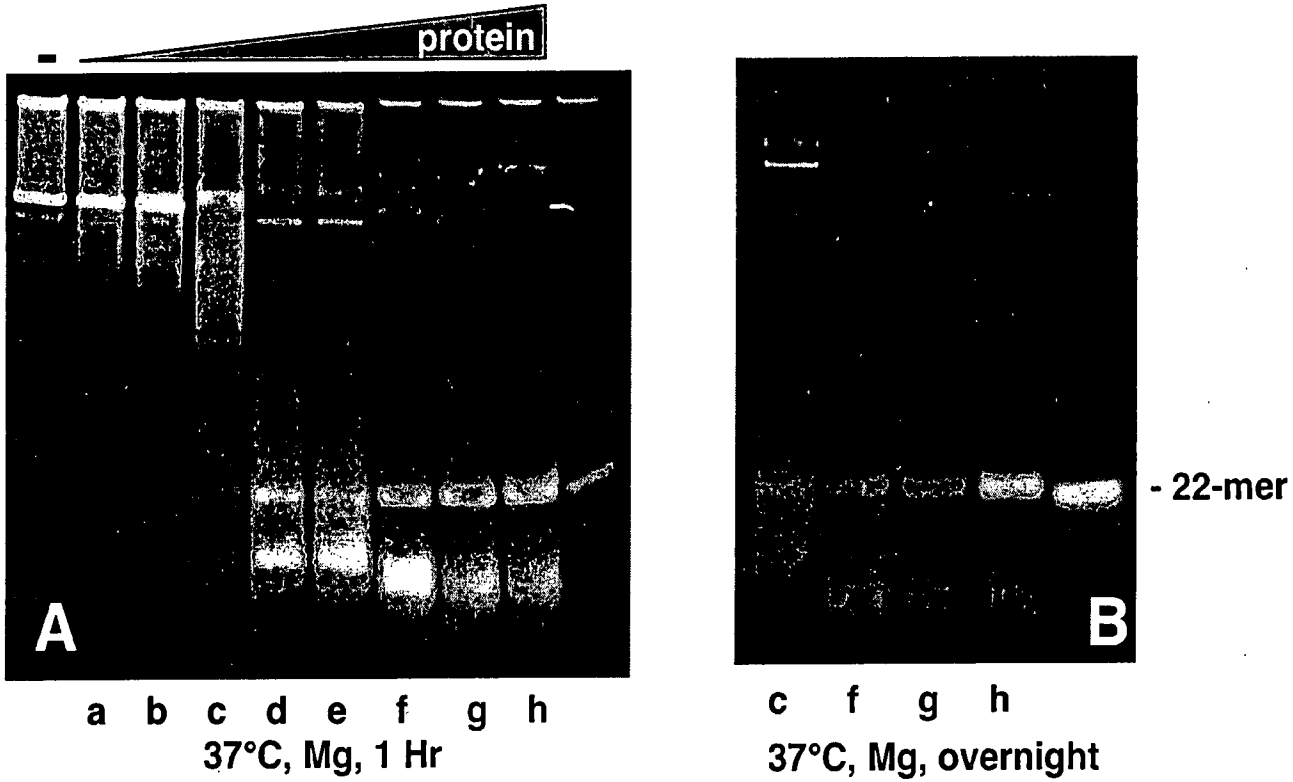

Figure 3

RNAse Activity of E38A

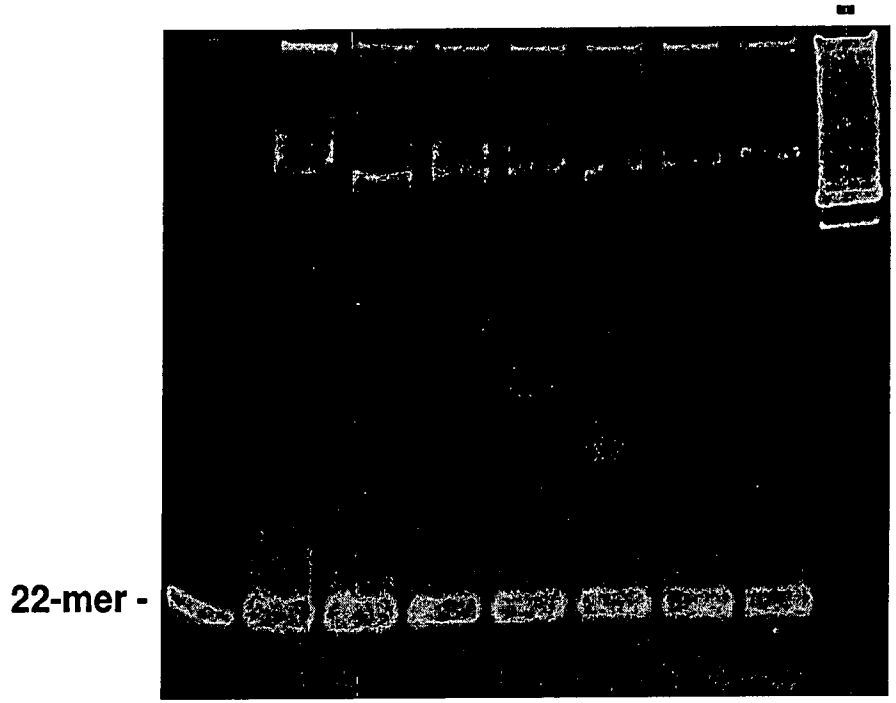

Figure 4

RNAse Activity of E38A

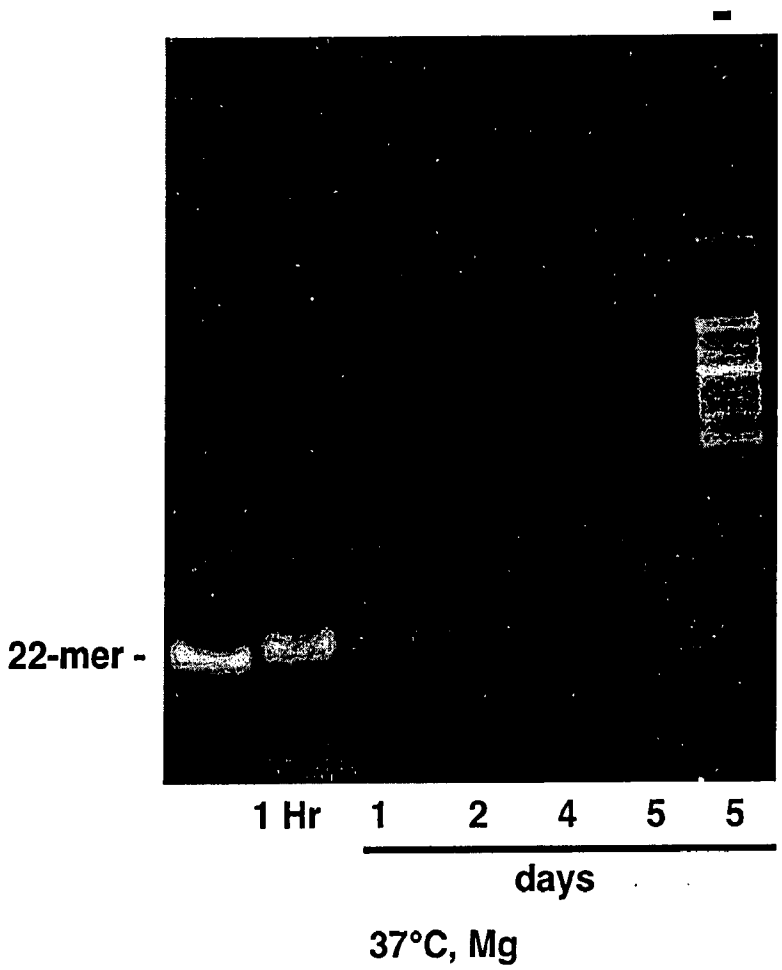

Figure 5

RNAse Activity of E38A

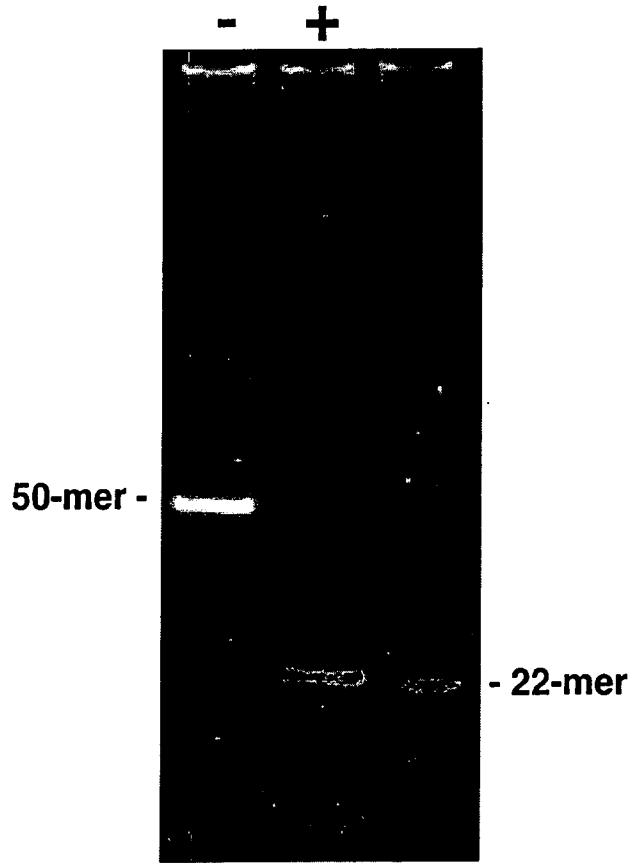

Figure 6

RNAse Activity of E65A

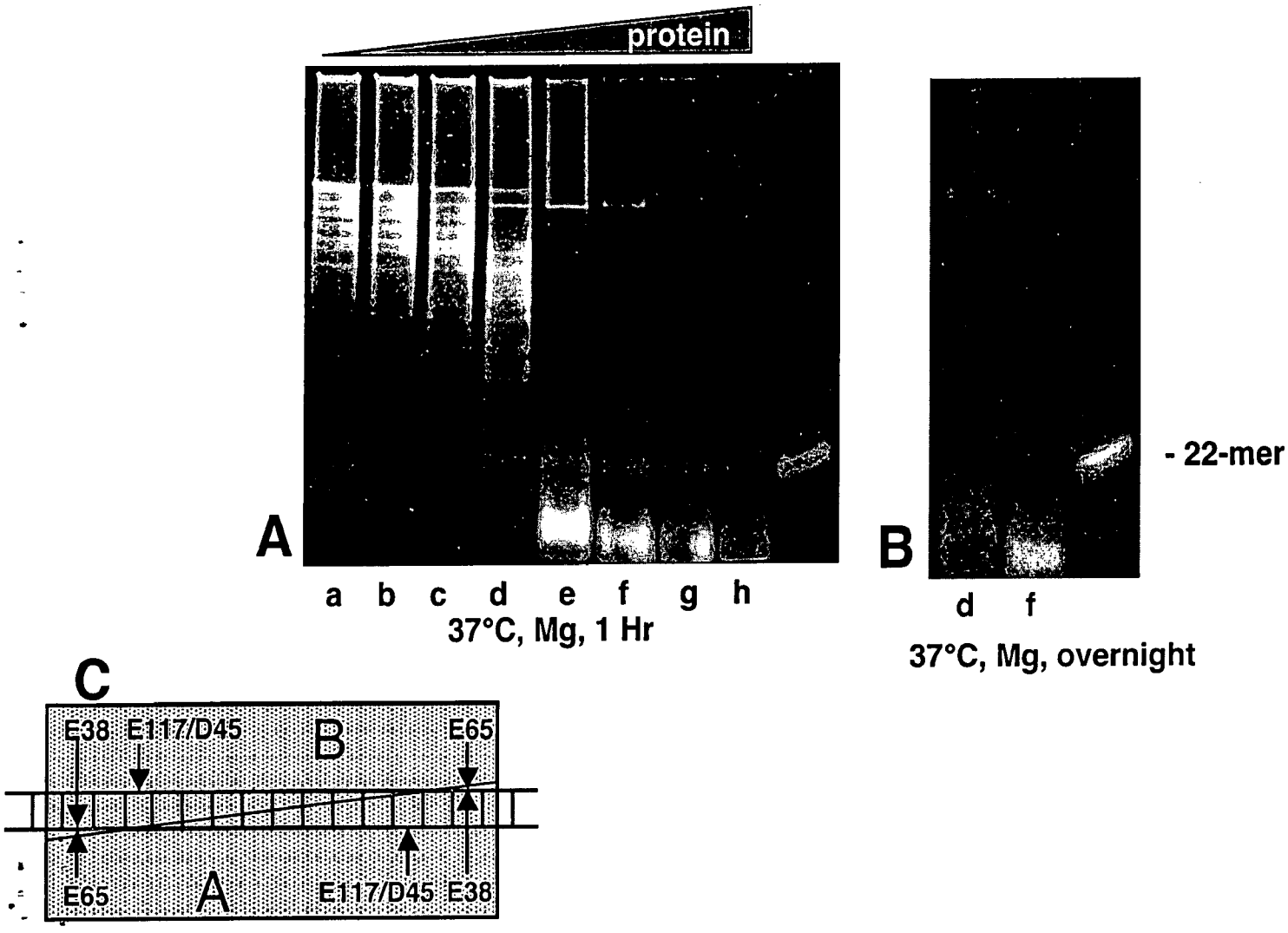

Figure 7

Side-by-Side

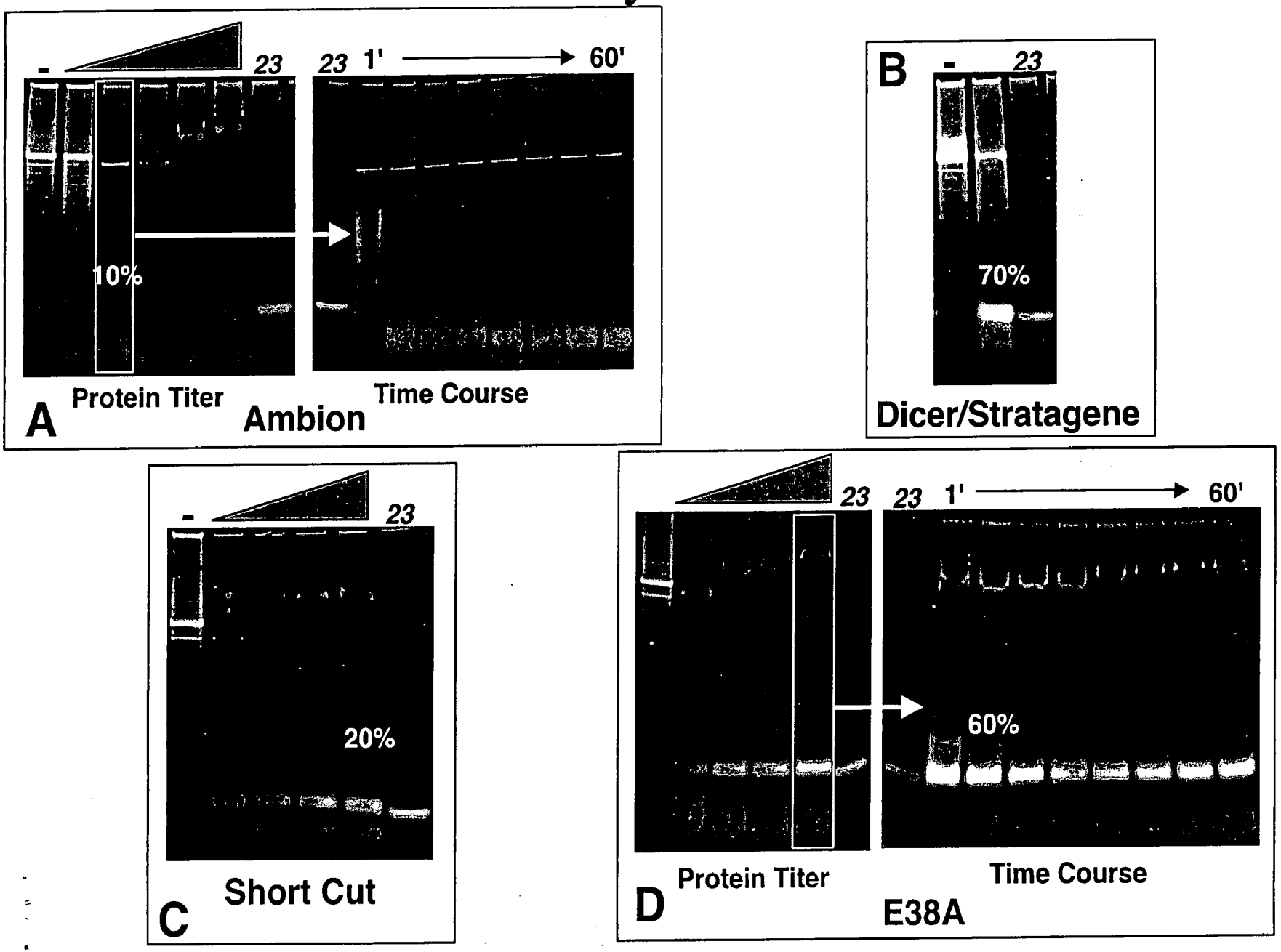

Figure 8

Summary of Mutant Analysis

WT	No Activity	23-mer
E38Q (wt)	**D45A (-)**	**E38A**
D45V* (-)	**E117D**	**E65A (-)**
E65P (-)		
E38A, E65A*		
E38Q, E65P		

Structure, 9, p. 1225, (2001)